

Identification of copy number variants associated with BPES-like phenotypes

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Abstract Blepharophimosis–Ptosis–Epicanthus inversus syndrome (BPES) is a well-characterized rare syndrome that includes an eyelid malformation associated with (type I) or without premature ovarian failure (type II). Patients with typical BPES have four major characteristics: blepharophimosis, ptosis, epicanthus inversus and telecanthus. Mutations in the *FOXL2* gene, encoding a forkhead transcription factor, are responsible for the majority of both types of BPES. However, many patients with BPES-like features, i.e., having at least two major characteristics of BPES, have an unidentified cause. Here, we report on a group of 27 patients with BPES-like features, but without

an identified genetic defect in the *FOXL2* gene or flanking region. These patients were analyzed with whole-genome high-density arrays in order to identify copy number variants (CNVs) that might explain the BPES-like phenotype. In nine out of 27 patients (33%) CNVs not previously described as polymorphisms were detected. Four of these patients displayed psychomotor retardation as an additional clinical characteristic. In conclusion, we demonstrate that BPES-like phenotypes are frequently caused by CNVs, and we emphasize the importance of whole-genome copy number screening to identify the underlying genetic causes of these phenotypes.

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Introduction

Blepharophimosis and ptosis are a reduction in the horizontal and vertical dimensions of the palpebral fissures respectively (Guercio and Martyn 2007). From 135 syndromes in the London Dysmorphology Database with blepharophimosis and ptosis, six are known to be associated with a chromosomal microdeletion (Winter and Baraitser 2008). In addition, a recent literature review illustrates that chromosomal aberrations are probably the most important underlying cause in patients with blepharophimosis and mental retardation phenotypes (Bartholdi et al. 2008). Only a few blepharophimosis syndromes have known causative genes: the Schwartz–Jampel syndrome (OMIM 255800), Freeman Sheldon syndrome (OMIM 193700), Hereditary neuralgic amyotrophy syndrome (HNA; OMIM 162100) (Laccone et al. 2008) and Blepharophimosis–Ptosis–Epicanthus inversus syndrome (BPES) (OMIM 110100). BPES is a rare autosomal dominant disorder characterized by a dysplasia of the eyelids. There are two types of clinical presentation: type I is associated with premature ovarian failure, whereas type II has no associated symptoms (Zlotogora et al. 1983). The typical BPES phenotype is characterized by four major features blepharophimosis, ptosis, epicanthus inversus (a small skin fold which arises from the lower eyelid and runs inwards and upwards) and telecanthus (increased distance between the medial lid margins). Haploinsufficiency of the *FOXL2* gene, encoding a putative forkhead transcription factor located at chromosome 3q23, is responsible for both types of BPES (Crisponi et al. 2001; De Baere et al. 2001, 2003). Using a combined mutation detection approach, it is possible to reveal the causal genetic defect in 88% of typical BPES patients. Molecular defects include chromosomal rearrangements (2%), intragenic mutations (81%) and genomic rearrangements comprising both deletions encompassing *FOXL2* (12%) and deletions located outside its transcription unit (5%) (Beysen et al. 2005, 2008). The lack of a detectable genetic *FOXL2* defect might be explained by several grounds, including subtle extragenic rearrangements, epigenetic changes and referral bias by the different clinicians (Beysen et al. 2008). Here, we focus on series of 27 patients with BPES-like features (i.e., having at least two main characteristics of typical BPES), who tested negative for *FOXL2* mutations and deletions of the *FOXL2* region, and in whom the causal defect remains to be elucidated (Beysen et al. 2008).

Novel whole-genome array-based technologies can detect copy number variants (CNVs) at much higher resolution than conventional cytogenetic methods, and hence might reveal CNVs that were previously unidentified. These techniques have been used with considerable success to reveal de novo CNVs in several phenotypes, including mental retardation and dysmorphism (Stankiewicz and

Beaudet 2007; Slavotinek 2008). Yet, the association between CNVs and BPES-like phenotypes has not been investigated. We performed whole-genome high-density arrays in the group of 27 *FOXL2* mutation-negative patients with a BPES-like phenotype in order to identify the underlying genetic cause explaining the phenotypes.

Methods

Patients

Genomic DNA was obtained from 27 patients with a BPES-like phenotype, being patients for whom at least two of the four diagnostic criteria of BPES (i.e., blepharophimosis, ptosis, epicanthus inversus and telecanthus), were mentioned on a clinical questionnaire. In 11 of the patients psychomotor retardation was identified as an additional clinical feature. Furthermore, all patients were clinically assessed by different clinicians. The patients were recruited in three genetic centers: (1) 22 patients from the Center for Medical Genetics, Ghent University Hospital, Belgium. Genomic patient DNA was used that was available from previously approved mutation studies (De Baere et al. 2001, 2003; Beysen et al. 2005, 2008), (2) three families from the Department of Clinical Genetics, Amsterdam Medical Centre, The Netherlands, (3) two patients from the Department of Clinical Genetics, Leiden University Medical Center, The Netherlands.

All patients were initially referred for *FOXL2* screening. Intragenic *FOXL2* mutations and copy number variants of the *FOXL2* gene and surrounding region were excluded in all patients by sequencing of the coding region and multiplex ligation-dependent probe amplification (MLPA) (P054, MRC Holland). In addition, conventional karyotyping was performed in 13 patients and considered normal.

Single nucleotide polymorphism arrays

Affymetrix GeneChip Human Mapping 262 K Nspl. This array was used for one patient and his parents. This SNP array contains ~262,000 25-mer oligonucleotides with an average spatial resolution of ~12 kb. An amount of 250 ng DNA was processed according to the manufacturer's instruction (<http://www.affymetrix.com>). SNP copy number was assessed in the patient using DNA-Chip Analyzer (dChip) software (version release 02-16-06) (Li and Wong, 2001).

Illumina's Sentrix HumanHap300 Genotyping Beadchip This platform was used for 23 patients. This array contains ~317,000 TagSNPs with a mean spatial resolution of approximately 9 kb. A total of 750 ng DNA was processed according to the manufacturer's instruction

(<http://www.illumina.com>). SNP copy number (logRratio) and B allele frequency were assessed in the patients using BeadStudio Version 3.2 (Illumina, Inc.).

Illumina's HumanCNV370-Duo BeadChip. For three patients this platform was used. This array contains ~317,000 TagSNPs and 52,000 non-polymorphic markers to specifically target nearly 14,000 known copy number variants with a mean spatial resolution of approximately 7.7 kb. The experiment was performed as described above. SNP copy number and B allele frequency were assessed in BeadStudio, Version 3.2 (Illumina, Inc.).

Evaluation of CNVs

Based on in-house validation studies deletions of at least five adjacent SNPs or with a minimum size of 150 kb and duplications of at least seven adjacent SNPs or with a minimum size of 200 kb were considered significant. All CNVs identified in this study were assessed by screening them against the Database of Genomic Variants (DGV) (<http://projects.tcag.ca/variation/>) and against our in-house available reference set of approximately 1,000 individuals. All regions that significantly overlapped with known polymorphic CNVs were excluded from further research.

MLPA

MLPA experiments were carried out to validate the presence of deletions and duplications identified by the arrays. When an aberration was confirmed by MLPA, the same probe set was used to perform segregation analysis in the parents (if available). At least two synthetic MLPA probes were designed within the aberration and MLPA experiments were performed as described (White et al. 2004). Probes were commercially obtained from Biolegio (Malden, The Netherlands). Amplification products were identified and quantified by capillary electrophoresis on an ABI 3130 genetic analyzer (Applied Biosystems, Nieuwerkerk aan de IJssel, The Netherlands). Fragment analysis was performed with the GeneMarker Software V1.51 (SoftGenetics, USA). Thresholds for deletions and duplications were set at 0.75 and 1.25, respectively (White et al. 2004).

Fluorescent in situ Hybridization analysis (FISH)

Fluorescent in situ Hybridization analysis was carried out by standard procedures as described (Dauwerse et al. 1990). FISH analysis was performed to verify the imbalances found with the array experiments and to exclude more complex rearrangements. BAC clones mapping to the

unbalanced chromosome regions were selected based on their physical location within the affected region (<http://www.ensembl.org>; Ensembl release 48 December 2007).

Gene prioritisation

The software tool Anni 2.0 (<http://www.biosemantics.org/Anni>) was used to search for potential candidate genes for BPES-like features in the CNVs found. For each gene a profile of related concepts is constructed that summarizes the context in which the gene is mentioned in the literature. Genes associated with similar topics are identified by hierarchical clustering of the corresponding gene concept profiles. The software was used according to the software's manual (Jelier et al. 2007).

Results and discussion

Identification and confirmation of 12 significant CNVs

In this study we included 27 patients based on the following criteria: (1) BPES-like phenotype (i.e., 2 or more major features of BPES, with or without additional dysmorphic features and psychomotor retardation), (2) exclusion of a *FOXL2* mutation or deletion of the *FOXL2* region. These patients were analyzed with whole-genome high-density arrays with an average spatial resolution of approximately 10 kb, in an attempt to identify the underlying genetic cause and to reveal potential novel candidate loci and genes for BPES-like phenotypes.

In total we identified 12 CNVs not previously described in phenotypically normal individuals according to the DGV and our in-house reference set, in nine out of 27 patients (33%). An overview of these changes is provided in Table 1. We found six duplications and six deletions. In three patients (cases 7, 8 and 9) a deletion as well as duplication was identified, suggesting the presence of an unbalanced translocation. The identified CNVs vary in size from 485 kb to 21.9 Mb. Table 2 represents the available clinical features from eight out of the nine patients carrying chromosome imbalances. Four of these patients display psychomotor retardation as an additional clinical feature.

Each of the 12 CNVs was confirmed by a second independent technique, either MLPA (patients 1–3, 5, 7–8), FISH (patients 4, 9) or another SNP array platform (patient 6). In eight of nine cases parental DNA was available for segregation analysis. MLPA and FISH analysis in these cases determined that chromosomal aberrations arose de novo in five out of eight individuals (patients 1–4, 8).

Table 1 CNVs detected by whole-genome high-density SNP arrays

ID	Gain/loss	Chromosome	Starting probe ^a	Ending probe ^b	Size of CNV ^c	Array	Confirmation	Status of inheritance	Number of genes
1	Loss	18q12.2q21.1	rs1693336	rs2576050	14.3 Mb	II	MLPA	de novo	18
2	Loss	17q21.31q21.31	rs393152	rs2016730	485 kb	II	MLPA	de novo	3
3	Gain	16p13.3p13.3	rs11077336	rs2239316	1.1 Mb	II	MLPA	de novo	21
4	Loss	10p12.33p12.31	rs17140331	rs11012683	4.5 Mb	I	FISH	de novo	7
5	Gain	11p15.4p15.4	rs7117223	rs4237772	545 kb	II	MLPA	Maternal	5
6	Gain	9q34.1q34.2	rs3012755	rs2027963	1.4 Mb	II	I	Maternal	23
7	Gain	10q25.2	rs10885243	rs9630042	21.9 Mb	II	MLPA	NA	61
	Loss	11q24.3	rs1940363	rs4508216	5 Mb	II	MLPA	NA	18
8	Gain	3q29	rs1021662	rs12639242	4 Mb	II	MLPA	de novo	43
	Loss	13q33.3	rs7321084	rs7316983	5 Mb	II	MLPA	de novo	36
9	Loss	3p25.3	rs9681213	rs3774210	8.6 Mb	II	FISH	Paternal ^d	12
	Gain	6p24.3	rs6596796	rs927402	7.7 Mb	II	FISH	Paternal ^d	23

I Affymetrix GeneChip Human Mapping 262 K *NspI*, II Illumina's Sentrix HumanHap300 Genotyping Beadchip, NA parental DNA not available

^a First aberrant probe

^b Last aberrant probe

^c Minimal size of aberration

^d Paternal inheritance can be concluded from the pedigree (see Fig. 3c)

De novo single CNVs

The CNVs identified in cases 1 and 2 were recently described in patients with BPES-like features, developmental delay and other dysmorphic features (Schinzel et al. 1991; Tinkle et al. 2003; Shaw-Smith et al. 2006; Koolen et al. 2006; Koolen et al. 2008).

Patient 1 had an interstitial deletion of 18q12.2q21.1 (14.3 Mb), and displayed blepharophimosis, ptosis, epicanthus inversus and mild dysmorphic features (abnormal nose and ears). The patient died at the age of 4. Several cases have been described with del(18)(q12.2q21.1) showing a consistent clinical pattern of mild dysmorphic features, blepharophimosis, obesity, mental retardation, seizures, behavioral problems and lack of major congenital anomalies (Schinzel et al. 1991; Tinkle et al. 2003).

In patient 2 an interstitial microdeletion of 17q21.31 (485 kb) was found (Fig. 1), overlapping with the region that was recently described in the new microdeletion syndrome 17q21.31 (Shaw-Smith et al. 2006; Koolen et al. 2006). This new microdeletion syndrome was originally identified by high-resolution genome analyses in patients with unexplained mental retardation, and was recently further characterized clinically and molecularly (Koolen et al. 2008). The deletions are associated with a common inversion polymorphism. Individuals carrying this deletion display a recognizable phenotype of blepharophimosis, a characteristic long face with pear shaped nose, large ears, hypotonia and mental retardation. Interestingly, the pheno-

type of patient 2, characterized by blepharophimosis, bilateral ptosis, epicanthus inversus, telecanthus, broad pear shaped nose, large ears, central and peripheral hypotonia and psychomotor retardation, is in agreement with this recognizable 17q21.31 microdeletion syndrome.

Patient 3 had a small interstitial duplication of 16p13.3 (1.1 Mb). To our knowledge, two patients have been reported with a partially overlapping duplication of this region, including a patient with an interstitial duplication of 345–480 kb on 16p13.3, encompassing the *TRAP1* and *CREBBP* genes that are also duplicated in patient 3 (Thienpont et al. 2007). The second patient was reported to have a duplication of 16p13.3 with a minimal and maximal size of 4.5 and 7 Mb, respectively (de Ravel et al. 2005). The duplicated region of patient 3 overlaps over at least 620 kb with the reported duplications. However, the two reported patients display no BPES-like features, suggesting that the region of approximately 600 kb that does not overlap is responsible for the BPES-like features in patient 3.

In patient 4 we detected a novel rearrangement, being an interstitial deletion of chromosome 10p12.33p12.31 (4.5 Mb) (Fig. 2). It was shown to occur de novo and it is therefore likely to be causative. The clinical features of this patient include blepharophimosis, ptosis, epicanthal folds, ectropion of the lower eyelid, S-shaped upper eyelid, synophrys, high palate, broad hands and feet, and an atrial septal defect type II. This patient has a normal psychomotor development.

Table 2 Clinical characteristics of BPES-like patients with significant CNVs

	1	2	3 ^a	4	5	6	7	8	9
BPES features									
Blepharophimosis	+	+	NA	+	+	–	+	+	+
Ptosis	+	+	NA	+	+	+	+	+	+
Epicanthus inversus	+	+	NA	–	–	+	+	+	–
Telecanthus	–	+	NA	+	–	–	+	+	–
Additional features									
Menstrual abnormalities	Male	Male	Male	Male	–, age?	–, 15 years	+, 19 years	–, 7 years	–, 43 years
Psychomotor retardation	–	+	NA	–	–	–	+	+	+
Growth retardation	–	–	NA	–	–	–	+	–	–
Microcephaly	–	–	NA	–	–	–	+	+	–
Abnormal ears	+	+	NA	–	+	–	+	+	–
Abnormal nose	+	+	NA	–	+	–	+	–	–
Flattened and broad nose	+	+	NA	–	–	+	+	–	+
Skeletal anomaly	–	+	NA	–	–	–	–	+	–
Syndactyly	–	–	NA	–	–	–	–	+	–
Clinodactyly	–	–	NA	–	–	–	–	+	–
Other	–	Central and peripheral hypotonia, pectus excavatum,	NA	Atrial septal defect type II	Reduced iris pigmentation, mild Brushfield spots	–	Midface hypoplasia, round face, narrow palate	Brachycephaly, diastasis recti	–

NA not available

^a Patient shows at least two characteristics of typical BPES and other features are unknown

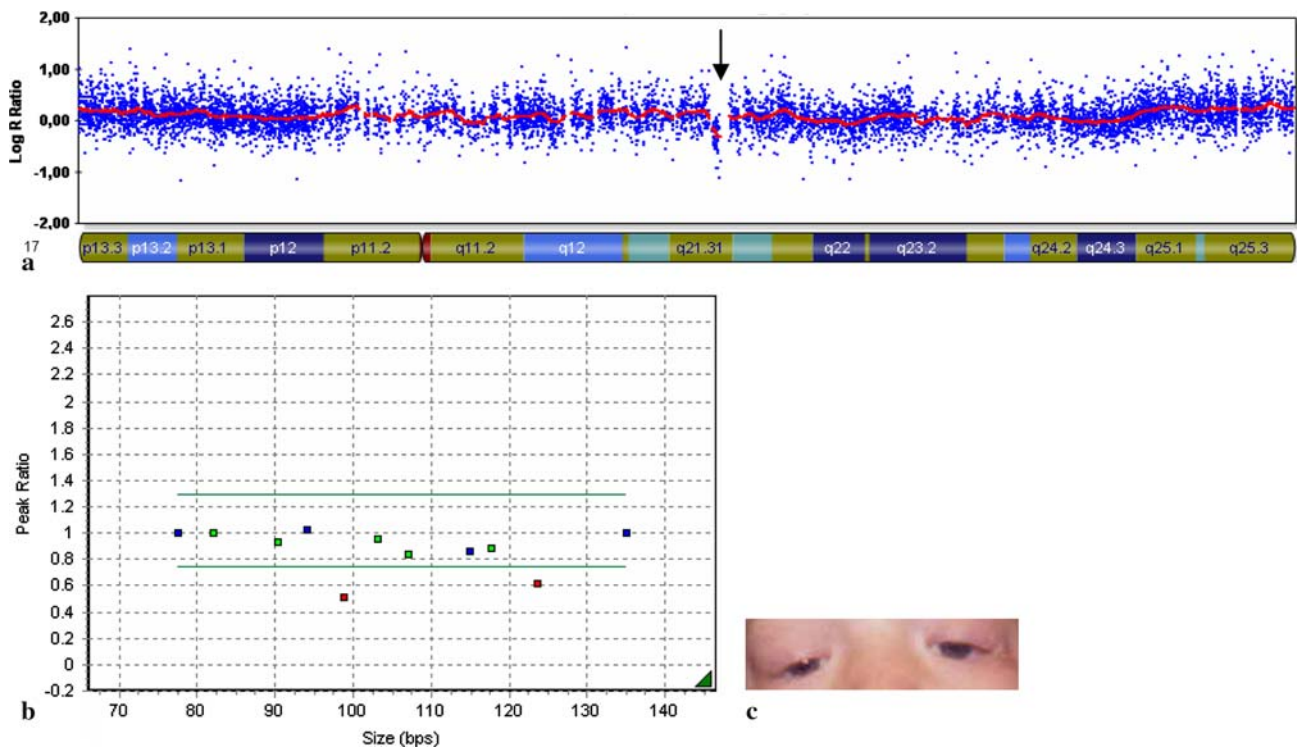


Fig. 1 Case 2. **a** Deletion of chromosome 17q21.31 (485 kb) found with the Illumina's Sentrix HumanHap300 Genotyping Beadchip. **b** MLPA analysis confirmed the deletion. Deleted probes: *MAPT*

and *KIAA1267*. **c** Picture of the eyes of case 2, characterized by blepharophimosis, bilateral upper lid ptosis, epicanthus inversus and telecanthus

Inherited single CNVs

In patient 5 a small interstitial duplication of 545 kb on chromosome 11p15.4 was detected. This gain was not reported as a variant in the DGV, but was found in the phenotypically normal mother of this patient, probably suggesting the absence of a causal relation between the duplication and the BPES-like phenotype in the child.

Furthermore, in case 6 an interstitial duplication of chromosome 9q34.1q34.2 (1.4 Mb) was found that was not described in the DGV. The mother of this patient was shown to carry the same duplication. The phenotype of patient 6 is characterized by bilateral ptosis, mild epicanthus inversus, horizontal nystagmus and high hypermetropia, while her mother displayed a mild ptosis and strabismus for which she underwent surgery. The occurrence of a phenotype in mother and child might suggest a causal relationship of the novel duplication and the phenotype.

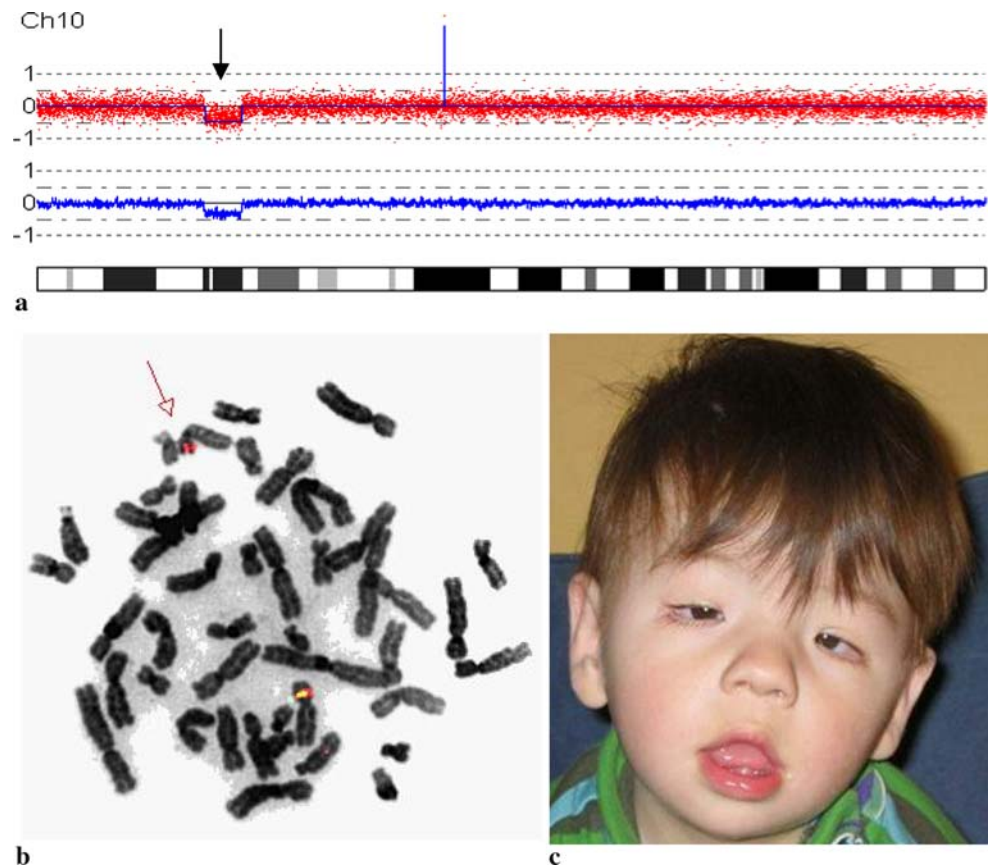
Complex CNVs

In three cases more complex rearrangements were identified (cases 7, 8 and 9). In these patients both duplication and deletion were found on different chromosomes, suggesting the occurrence of an unbalanced translocation (Table 1). Patient 7 was found to carry a terminal duplica-

tion of chromosome 10q (21.9 Mb) and a terminal deletion of chromosome 11q (5 Mb). Recently, a patient was described with approximately the same duplication of chromosome 10q and a deletion located on chromosome 4q (Bartholdi et al. 2008). This patient displayed the following clinical features: blepharophimosis, ptosis, epicanthus inversus, downslanting palpebral fissures, generalized hypotonia and developmental delay. The phenotype was ascribed to the duplication of chromosome 10q (Bartholdi et al. 2008). Our patient 7 showed a similar phenotype; therefore, we hypothesize that the duplication of chromosome 10q might contribute to the BPES-like phenotype in this patient. For patient 7 it could not be confirmed whether this complex rearrangement arose from a balanced translocation in one of the parents.

Patient 8 showed a terminal duplication of chromosome 3q (4 Mb) and a terminal deletion of chromosome 13q (5 Mb). FISH analysis revealed the presence of an unbalanced translocation in the patient and showed no abnormalities in the parents. This patient showed a BPES-like phenotype and some additional dysmorphic features. In the ECARUCA database 28 cases were listed with a del(13)(q33.3q34). In 4/28 blepharophimosis was described as a clinical feature, in 3/28 ptosis, and in 2/28 telecanthus. No association between BPES-like features and 3q29 could be found in the literature.

Fig. 2 Case 4. **a** Deletion of chromosome 10p12.33-p12.31 (4.5 Mb) detected with Affymetrix GeneChip Human Mapping 262 K *NspI*. **b** FISH analysis confirmed the deletion. Control probe: RP11-390B4 (*red*), deleted probe 10p12.31: RP11-177H22 (*green*). **c** Facial picture of case 4, showing blepharophimosis, ptosis, epicanthal folds, ectropion of the lower eyelid, S-shaped upper eyelid, synophrys



In patient 9 a terminal deletion of chromosome 3p (8.6 Mb) and a terminal duplication of chromosome 6p were observed (7.7 Mb) (Fig. 3a). Her facial appearance is characterized by blepharophimosis, ptosis, flattened and broad nose, long philtrum (IV-2) (Fig. 3d). In addition she displays psychomotor retardation. Patients with a terminal deletion of the short arm of chromosome 3 have been described to display blepharophimosis, epicanthus, upturned nose, long philtrum, microcephaly and polydactyly (Malmgren et al. 2007). Both the terminal deletion of 3p and terminal duplication 6p are associated with BPES-like features according to literature (Schinzel 2001). With standard G-banding the deletion and duplication were not visible. FISH with DNA probes specific for the subtelomeric region of chromosome 3p and 6p revealed that this rearrangement arose by an unbalanced translocation between chromosome 3p and 6p (Fig. 3b). Furthermore, another patient of this family with similar clinical features was found to have the same unbalanced rearrangement using SNP array analysis (III-5) (Fig. 3d). Subsequently, segregation analysis of the 3;6 translocation was performed in other members by FISH analysis (Fig. 3c), and revealed a familial 3;6 translocation. An unbalanced translocation, resulting in a terminal duplication of chromosome 3p and a terminal deletion of chromosome 6p, was observed in one family member (V-2). This patient showed no blepharophi-

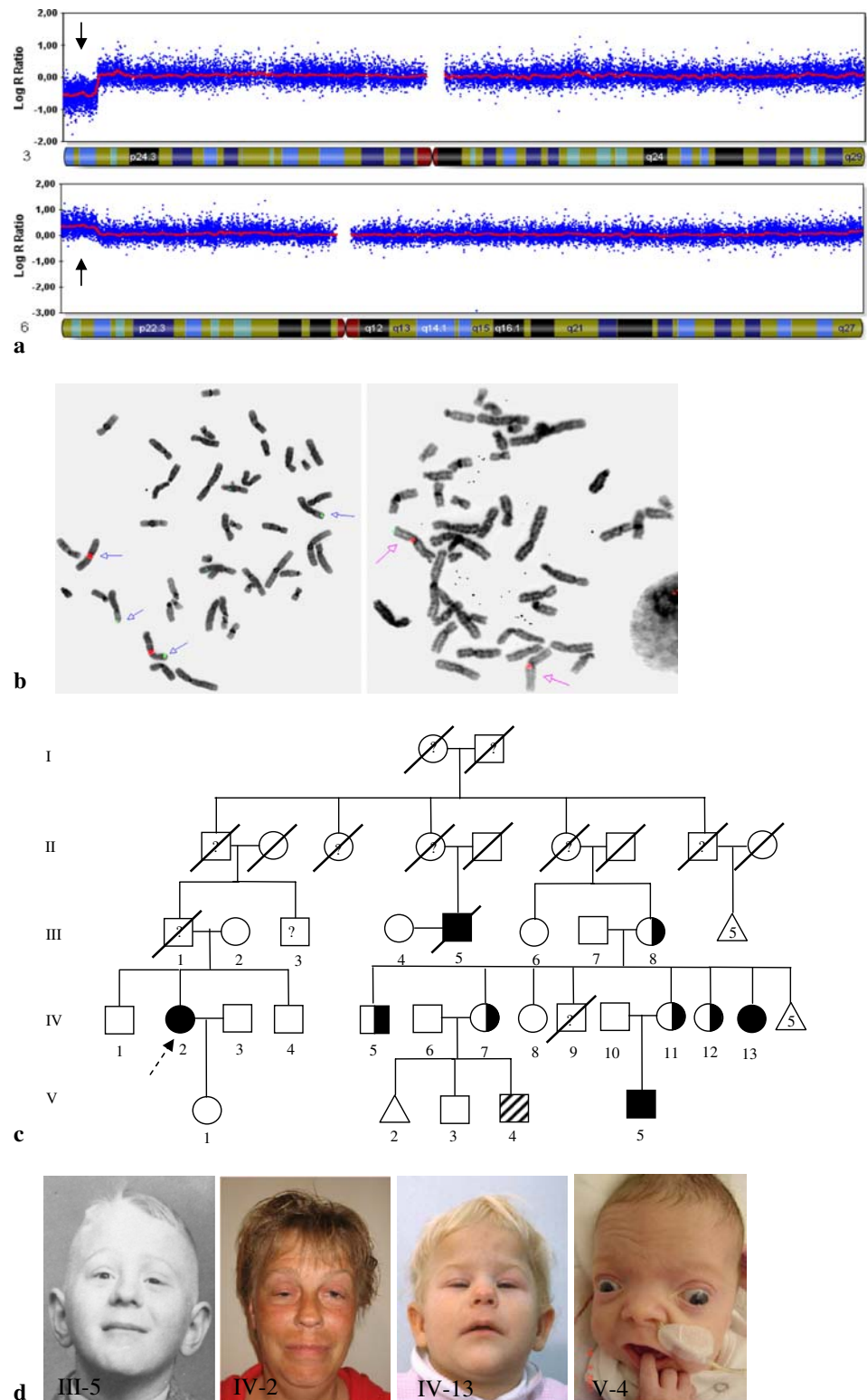
mosis, but displayed macrocephaly, Dandy–Walker malformation, hypoplasia of the cerebellum, hypertelorism, divergent strabismus, broad and flat nasal bridge and low set ears (Fig. 3d).

Previously performed conventional karyotyping revealed no chromosome abnormalities in patients 7, 8 and 9. These findings demonstrate that even large chromosomal imbalances are not always easily detected with standard G-banding, emphasizing the importance of a whole-genome screen with a high resolution array technique.

Candidate gene analysis

Assuming that the identified CNVs are causative for BPES-like phenotypes, it can be postulated that the corresponding chromosomal regions might reveal some relevant candidate genes contributing to some specific features. Most of these rearrangements, however, are too large to allow pinpointing of major candidate genes. Despite this, we used the software tool Anni in order to find functional associations between large numbers of genes contained in the identified CNVs and biomedical information from literature (Jelier et al. 2007). However, for all these regions no significant candidate genes were found. The availability of more BPES-like patients and more subtle rearrangements may be useful in the search for candidate genes involved in BPES-like phenotypes.

Fig. 3 Case 9. **a** Terminal deletion of chromosome 3p25.3 (8.6 Mb) and terminal duplication of 6p24.3 (7.7 Mb) detected with the Illumina's Sentrix HumanHap300 Genotyping Beadchip. The derivative chromosome 3 was not observed by conventional G-banding. **b** FISH analysis revealed the presence of an unbalanced 3;6 translocation. *Left panel*, control probe: CEP3 (red) (Vysis), 6pter probe: GS-19615 (green); *right panel*, control probe: CEP3 (red) (Vysis), 3pter probe: GS-186B18 (green). **c** Pedigree of the family with the segregating $t(3;6)$ analyzed with FISH experiments. *half-filled square*: balanced $t(3;6)$; *filled square* 3p25.3 deletion and 6p24.3 duplication; *stripped square*: 3p25.3 duplication and 6p24.3 deletion. **d** Facial picture of case 9 (IV-2), characterized by blepharophimosis, ptosis, flattened and broad nose. Facial pictures of two relatives (III-5, IV-13) with the same unbalanced translocation as case 9 are presented. These members show similar clinical features. In addition, a facial picture of a relative (V-2) with the opposite unbalanced translocation is shown. No BPES-like features are seen in this child



General conclusion

BPES-like features are associated with many syndromes and chromosomal disorders. In this study novel rearrangements were identified in 33% of patients with BPES-like

phenotypes. These changes are presumed to contribute to the phenotype in most cases. Overall, we demonstrated that whole-genome high-density array screening is a powerful strategy to reveal the underlying genetic defect in BPES-like phenotypes. We conclude that patients with all four

main features of typical BPES should be tested first for genetic defects in *FOXL2*. However, patients with a BPES-like phenotype that can be distinguished from typical BPES, should be tested with a genome wide array prior to *FOXL2* genetic testing.

Electronic-database information

Affymetrix: <http://www.affymetrix.com>

Anni: <http://www.biosemantics.org/Anni>

Database of Genomic Variants: <http://projects.tcag.ca/variation/>

ECARUCA: <http://ecaruca.net/>

ENSEMBL: <http://www.ensembl.org/index.html>

Illumina: <http://www.illumina.com/>

MRC Holland: <http://www.mlpa.com/pages/indexpag.html>

Primer3: http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi

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